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Comparative nitric oxide production by LPS-stimulated monocyte-derived macrophages from *Ovis canadensis* and *Ovis aries*

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Abstract

Bighorn sheep are more susceptible to respiratory infection by *Mannheimia haemolytica* than are domestic sheep. In response to bacterial challenge, macrophages produce a number of molecules that play key roles in the inflammatory response, including highly reactive nitrogen intermediates such as nitric oxide (NO). Supernatants from monocyte-derived macrophages cultured with *M. haemolytica* LPS were assayed for nitric oxide activity via measurement of the NO metabolite, nitrite. In response to LPS stimulation, bighorn sheep macrophages secreted significantly higher levels of NO compared to levels for non-stimulated macrophages. In contrast, levels of NO produced by domestic sheep macrophages in response to *M. haemolytica* LPS did not differ from levels detected in non-stimulated cell cultures. Nitrite levels detected in supernatants of LPS-stimulated bighorn macrophage cultures treated with an inducible nitric oxide synthase (iNOS) inhibitor, N^G-monomethyl-L-arginine, were similar to that observed in non-stimulated cultures indicating a role for the iNOS pathway.
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Keywords: *Mannheimia haemolytica*; Lipopolysaccharide; Bighorn sheep; Domestic sheep; Macrophage; Nitric oxide

Résumé

Les moutons 'Bighorn' (*Ovis canadensis*) sont plus sensibles à l'infection respiratoire par *Mannheimia haemolytica* que les moutons domestiques. En présence de cette bactérie, les

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macrophages produisent un certain nombre de molécules qui jouent des rôles important dans la réponse inflammatoire, y compris les intermédiaires de pazote hautement tels que l'oxyde nitrique (NO). Les surnageants des macrophages ont été cultivés en présence de lipopolisaccharide de *M. haemolytica* pour mesurer la présence de poxyde nitrique. En réponse à la stimulation de lipopolisaccharide, les macrophages de moutons 'Bighorn' ont sécrété des niveaux d'oxyde nitrique sensiblement plus élevés que ceux obtenus à partir des macrophages non-stimulés. En revanche, les niveaux d'oxyde nitrique produits par des macrophages de moutons domestiques en réponse aux lipopolisaccharide de *M. haemolytica* n'ont pas différé par rapport aux niveaux détectés dans les cultures de cellules non-stimulées. Les niveaux de nitrite détectés dans les surnageants de culture de macrophage de 'Bighorn' stimulés par les lipopolisaccharide et traités avec un inhibiteur induisant la synthase d'oxyde nitrique, N^G-monométhyle-L-arginine, étaient semblables à ceux observés dans les cultures non-stimulées.
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Mots clés: Mannheimia haemolytica; Lipopolysaccharide; Moutons 'Bighorn' (*Ovis canadensis*); Moutons domestiques; Macrophage; Oxyde nitrique

1. Introduction

Pneumonia epizootics are frequent among herds of bighorn sheep (*Ovis canadensis*) and are likely an important reason for the decline of bighorn sheep numbers. Members of the genuses *Mannheimia* and *Pasteurella* seem to be common organisms associated with these epizootics [1]. Bighorn sheep are more susceptible to respiratory infections than domestic sheep. In fact, transmission of the respiratory pathogen *Mannheimia haemolytica* from domestic sheep (*Ovis aries*) to bighorn sheep [2] can have devastating consequences to bighorn sheep populations. The basis for the differences in susceptibility to specific pathogenic microorganisms between bighorn and domestic sheep remains to be elucidated.

Macrophages play a critical role in the initial defense of the lung against pathogens entering the lower airways. In response to pathogenic challenge, macrophages elaborate a number of molecules that play a key role in the innate immune response, including proinflammatory cytokines, chemokines, and highly reactive nitrogen and oxygen intermediates, such as nitric oxide (NO). NO is a free radical end product of the metabolism of L-arginine to L-citrulline by inducible NO synthase (iNOS). Furthermore, the NO pathway can yield other radicals (e.g. NO_2), stable anions (nitrites, nitrates), unstable higher oxides (e.g. N_2O_3) and unstable peroxides, [3] all of which may have bioregulatory activity. Thus, the NO pathway arms macrophages with several molecules that might be involved in the elimination of pathogenic microorganisms. Paradoxically, NO production in response to pathogenic challenge can cause host tissue damage [4,5] and suppression of beneficial host immune-mediated mechanisms [3].

Host species differences in NO production and iNOS activity are known to exist. Previous research has shown that pulmonary alveolar macrophages of domestic sheep produced low levels of NO. Furthermore, NO levels were not substantially increased by stimulation with bacterial LPS, interferon-γ, or an ovine lentivirus [6]. In the present study, we derived bighorn sheep macrophages from peripheral blood mononuclear cells.

Characterization of the derived cells as macrophages was based on morphology, staining for the myeloid lysosomal marker CD68, and cell-surface expression of CD14. Differences in susceptibility to *M. haemolytica* between bighorn and domestic sheep may be partially explained by a variation in the inflammatory response to this pathogen or to molecules elaborated by this organism. Given the potential role of NO in pathophysiology, the ability of bighorn sheep monocyte-derived macrophages to produce NO in response to *M. haemolytica* LPS was compared to that of macrophages from domestic sheep. The addition of an iNOS inhibitor, N^G-monomethyl-L-arginine was used to show that the nitrite levels measured were produced via the iNOS pathway.

2. Materials and methods

2.1. Peripheral blood monocyte isolation and in vitro culture

Blood samples were obtained from rocky mountain or desert bighorn sheep (*O. canadensis*) or from domestic sheep (*O. aries*). Bighorn sheep sampled were captive animals at the Idaho Department of Fish and Game, Caldwell, ID; the Living Desert Zoo, Palm Springs, CA; and Utah's Hogle Zoo, Salt Lake City, UT. Domestic sheep blood samples were obtained from a herd at the University of Idaho, Caine Veterinary Teaching Center, Caldwell, ID or from the National Animal Disease Center (NADC). For the present experiments, a total of seven domestic and 12 bighorn sheep blood samples were collected in tubes containing anticoagulant citrate-dextrose. Samples that were shipped to NADC were sent overnight at room temperature. For cell isolation, samples were centrifuged at $425 \times g$ for 30 min at 4 °C. Mononuclear cells were collected, contaminating RBCs lysed, and cells filtered through a 70 μm nylon mesh. Cells were resuspended in supplemented RPMI 1640 containing L-glutamine, HEPES, antibiotics, 10% FBS, and 10% domestic sheep serum, and incubated at 37 °C in 5% CO_2 atmosphere for 1 h. Supernatant containing non-adherent cells was aspirated and fresh medium added. Adherent mononuclear cells (2×10^6 per ml) were incubated at 37 °C for 2 weeks in T25 flasks (Corning, Inc., Corning, NY) or in glass chamber slides (Lab-Tek, Nalge Nunc International, Naperville, IL). Fresh supplemented medium was added every third day of the culture period. At the end of the culture period, cells cultured in glass chamber slides were washed and fixed in methanol for subsequent immunohistochemical analysis. Cells cultured in T25 flasks were harvested with a cell scraper, washed and counted. Macrophages were subsequently stained for flow cytometric analysis or were cultured in vitro in microtiter plates (4×10^5 per well) for 24 h with or without *Mannheimia* (*Pasteurella*) *haemolytica* LPS (10 μg/ml). *M. haemolytica* serotype A1 (bovine strain L101) rough LPS was prepared as previously described [7]. Endotoxic activity of the LPS preparation (1.8 EU/ng) was determined using the QCL-1000 chromogenic limulus amebocyte lysate test kit (Cambrex Bio Science Rockland, Inc., Rockland, ME). Following the 24 h culture period, samples of control and LPS-stimulated macrophages were examined by trypan blue exclusion.

2.2. Immunohistochemical staining for myeloid lysosomal marker CD68

EMB11, a mouse anti-human CD68 mAb (Dako, Carpenteria, CA) was utilized in an imunohistochemical assay for detection of cells of the myeloid lineage. This mAb has been used to label macrophages in sections of human, monkey, and bovine tissue [8–10]. Briefly, cells were incubated with primary antibody (1:25) overnight at 4 °C. In a previously published study, it was shown that this is an optimal concentration of EMB11 for immunocytochemical staining of myeloid-lineage cells [10] that does not result in significant background (non-specific) staining in tissue sections. Negative control sections were included in which no primary antibody was added. Peroxidase-conjugated goat anti-mouse IgG and 3′3-diaminobenzidine substrate (Vector Labs, Burlingame, CA) were used in subsequent steps. Sections were counter stained with Harris hematoxylin.

2.3. Flow cytometric analysis for macrophage differentiation antigen CD14

Cells were stained with a mouse IgG1 mAb which recognizes CD14 (CAM36A; VMRD) and has reported cross-reactivity for numerous species. Primary antibody diluted (1:150) in FACS buffer (PBS containing 0.02% sodium azide and 1% serum) was added and cells incubated for 15 min at 4 °C. An isotype-matched mouse IgG1 Ab was included as a negative control for staining. Cells were washed, appropriate secondary antibody added (1:100), and incubation was for 15 min at 4 °C. Cells were washed and fixed in 2% paraformaldehyde in PBS prior to subsequent analysis. Forward angle and orthogonal light scatter and fluorescence parameters were acquired on 10,000 events using a Becton Dickinson FACScan flow cytometer (San Jose, CA). Live cells were gated based on forward angle and orthogonal light scatter. Data were analyzed using CellQuest software (Becton Dickinson).

2.4. Nitric oxide assay

Nitrite is the stable oxidation product of NO. The amount of nitrite in culture supernatants is indicative of the amount of NO produced by cells in culture. Nitrite was measured using the Griess reaction [11] performed in 96-well microtiter plates (Immulon 2; Dynatech Laboratories, Inc., Chantilly, VA). Culture supernatant (100 μl) was mixed with 100 μl of Griess reagent (0.5% sulfanilamide; Sigma Chemical Co., St Louis, MO) in 2.5% phosphoric acid (Mallinkrodt Chemicals, Inc., Paris, KY) and 0.05% *N*-(1-naphthyl) ethylenediamine dihydrochloride (Sigma). The mixture was incubated at 21 °C for 10 min. Absorbencies of test and standard samples at 550 nm were measured using an automated ELISA plate reader (Molecular Devices, Menlo Park, CA). Samples were diluted in supplemented RPMI medium. Absorbencies of standards, controls, and test samples were converted to nanogram per millilitre of nitrite by comparison with absorbances of sodium nirite (Fisher Chemicals, Fair Lawn, NJ). N^G-monomethyl-L-arginine (L-NMMA; Calbiochem, La Jolla, CA), a competive inhibitor of the enzyme nitric oxide synthase (NOS) was added (1.15 nM; equimolar to the amount of L-arginine in the culture medium) to parallel cultures to verify that the nitrite produced was due to the activity of NOS. Three independent experiments were conducted and differences in nitrite levels between

treatment groups (in total, $n=7$ domestic sheep; $n=12$ bighorn sheep) were determined using Student's t test.

3. Results

3.1. Derivation and characterization of macrophages from O. canadensis

In the present study, macrophages were derived from peripheral blood monocytes isolated from bighorn sheep or domestic sheep. Adherent peripheral blood mononuclear cells were cultured in vitro for a 2 week period. At the conclusion of the in vitro culture period, Bighorn sheep peripheral blood monocyte-derived cells exhibited morphology typical of macrophages with numerous cytoplasmic processes (Fig. 1). Similar morphology was observed for cells derived from peripheral blood monocytes of domestic sheep (not shown).

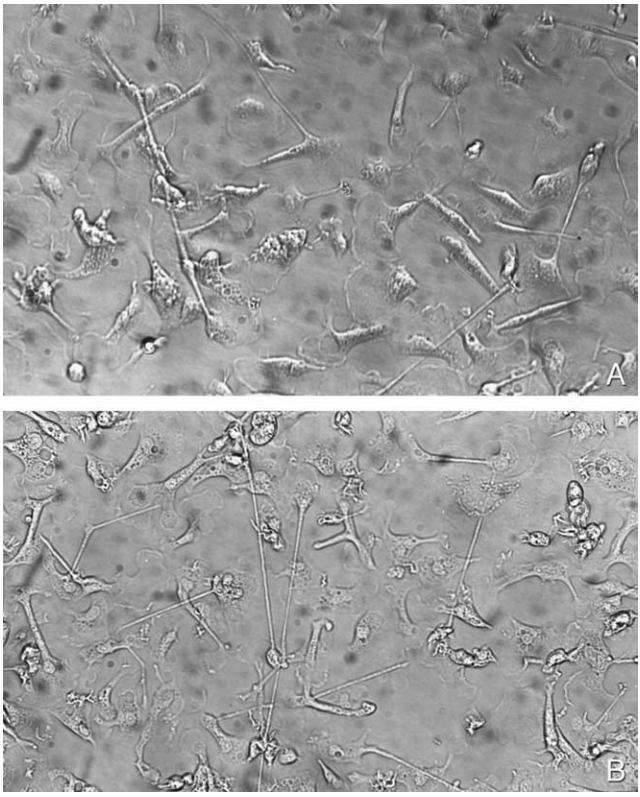


Fig. 1. Representative photomicrograph of bighorn sheep macrophages following 2 weeks culture. Blood samples were obtained from rocky mountain or desert bighorn sheep. Mononuclear cells were isolated and adherent cells obtained following an 1 h incubation at 37 °C. Adherent cells were cultured in vitro for 2 weeks in supplemented medium at 37 °C with 5% CO_2. Original magnification 20×.

EBM11, a murine anti-human CD68 mAb was utilized in an immunohistochemical assay to further characterize monocyte-derived bighorn sheep macrophages. CD68, a member of the scavenger receptor family, is a 110 kDa glycoprotein that has been used as a marker of myeloid lineage cells. As previously shown for other species [8–10], the antigen recognized by EBM11 is expressed primarily as an intracytoplasmic molecule associated with lysosomal granules in macrophages from both domestic and bighorn sheep (Fig. 2B and D, respectively).

For flow cytometric analysis of CD14 expression on monocyte-derived domestic or bighorn sheep macrophages, cells were stained with mAb CAM36A that has known cross-reactivity for numerous ungulate species (Fig. 3). In the present study, we observed variability between individual bighorn sheep in the percentage of peripheral blood-derived macrophages that expressed CD14 (range 82–92%). Overall, approximately 84% of the monocyte-derived bighorn sheep macrophages expressed CD14. Domestic sheep macrophages expressed CD14 at levels similar to that observed for bighorn sheep macrophages (Fig. 3A). Taken together, results from morphological, immunohistochemical and flow cytometric analyses indicate that the cells derived from bighorn sheep

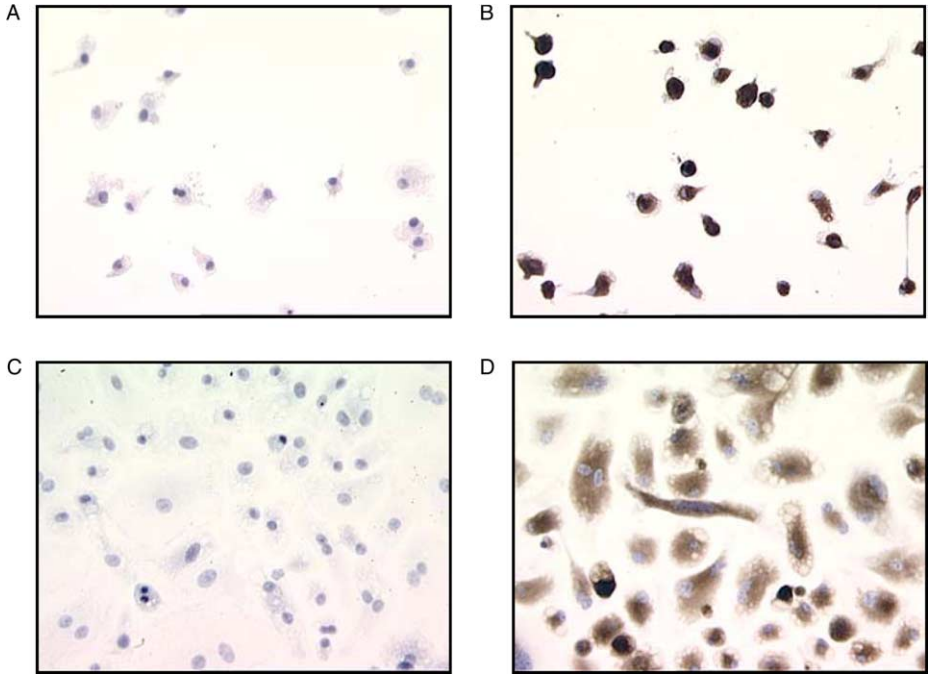
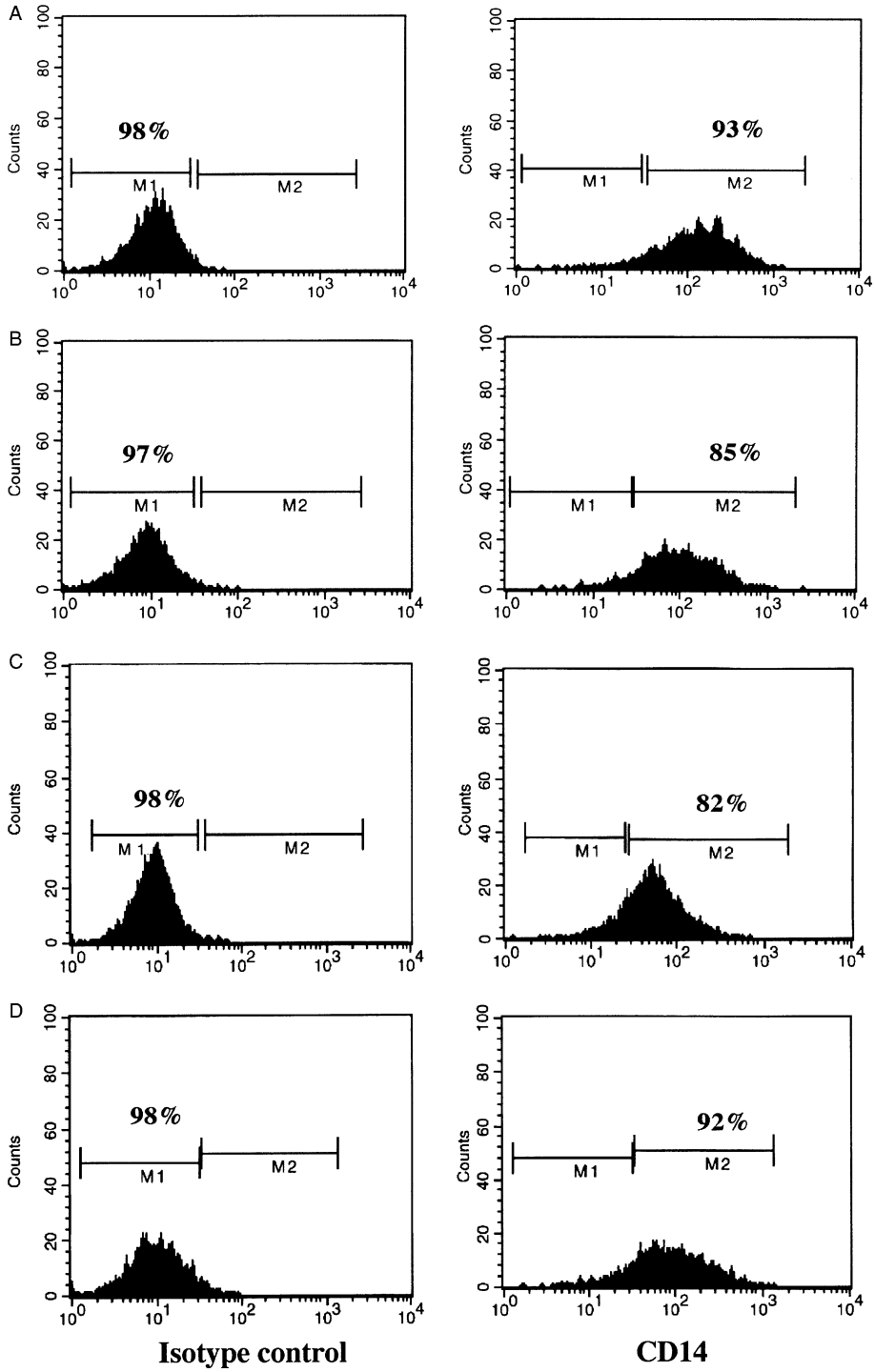


Fig. 2. Photomicrographs of macrophages stained with anti-CD68 mAb EBM11. Cells were cultured for 2 weeks in glass chamber slides, washed and fixed with methanol. Domestic sheep (B) or bighorn sheep macrophages (D) were incubated overnight with EBM11 mAb (1:25) at 4 °C. Peroxidase-conjugated goat anti-mouse IgG and 3′3-diaminobenzidine substrate were used in subsequent steps. Control sections from domestic sheep (A) and bighorn sheep (C) were included in which no primary antibody was added. Sections were counterstained with hematoxylin. Original magnification 40×.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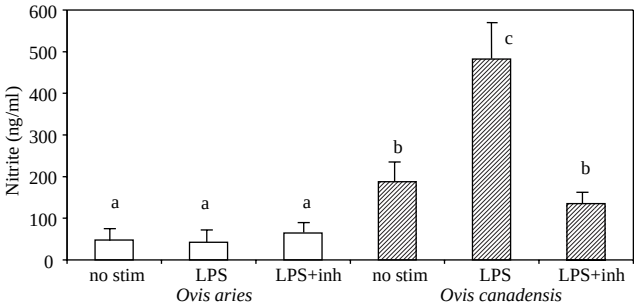

Fig. 4. Levels of nitrite (ng/ml) in culture supernatants from domestic sheep (*Ovis aries*) or bighorn sheep (*Ovis canadensis*) stimulated with *Mannheimia haemolytica* LPS (10 μg/ml) for 24 h. Nitrite was measured using the Griess reaction as described in the text. Absorbances of standards, controls and test samples were converted to nanogram per millilitre of nitrite by comparison with absorbances of sodium nitrite. N^G-monomethyl-L-arginine (L-NMMA), a competitive inhibitor of iNOS was added in parallel cultures to verify that the nitrite produced was due to the activity of iNOS. Mean (± SEM) between treatment groups within an ovine species with different letters (a, b, or c) are significantly different ($P < .05$). Data shown are from three independent experiments (in total, domestic sheep, $n = 7$; bighorn sheep, $n = 12$).

peripheral blood monocytes have characteristics consistent with those previously described for macrophages in other species.

3.2. Differential nitrite production

Finally, we examined the ability of bighorn sheep macrophages to produce NO in response to stimulation with *M. haemolytica* rough LPS (10 μg/ml) for 24 h. In preliminary experiments, we determined that 10 μg/ml was an optimal dose for stimulation of macrophages from domestic and bighorn sheep. For example, at 1 μg/ml *M. haemolytica* LPS did not induce nitrite secretion by either domestic or bighorn sheep macrophages over levels seen in non-stimulated cell cultures. Moreover, this dose is the same as used in previously published experiments for *M. haemolytica* LPS stimulation of bovine macrophages [12]. Finally, we noted no toxicity to the cells at this dose of LPS when comparing LPS-stimulated to non-stimulated macrophages. The amount of nitrite within culture supernatants was used as an indication of the amount of NO produced by cells in culture. As shown in Fig. 4, the addition of rough LPS to the bighorn sheep macrophages induced a significant ($P < .05$) increase in the levels of nitrite (484 ng/ml) produced in comparison to the non-stimulated bighorn sheep macrophage cultures (187 ng/ml). No significant differences were noted in the levels of nitrite secreted by desert or rocky mountain bighorn sheep macrophages in response to LPS. The addition of the

Fig. 3. Expression of CD14 on domestic or bighorn sheep macrophages. Peripheral blood mononuclear cells were isolated and adherent cells were cultured for 2 week. Following the culture period, cells were stained for flow cytometric analysis as described in the text. Shown are histograms from individual domestic (A) or rocky mountain (B and D) or desert bighorn sheep (C) gated on live cells based on forward versus side scatter characteristics. The data shown are representative of three independent experiments ($n = 4$ domestic sheep and $n = 7$ bighorn sheep).

iNOS inhibitor, L-NMMA, reduced the levels of nitrite to those levels observed for non-stimulated cultures. In agreement with previous research [6], nitrite levels produced by domestic sheep macrophages following *M. haemolytica* LPS stimulation did not differ from levels observed for non-stimulated controls.

4. Discussion

It has been estimated that ancestors of bighorn and domestic sheep diverged more than 2 million years ago. Ancestors of modern bighorn sheep are thought to have survived in a southern clime during the last ice age and later repopulated the North American continent [13]. There are a number of different theories regarding the origins of domestic sheep. However, modern domestic sheep likely originated from ancestral sheep of Europe and Asia. Bighorn sheep numbers have been in decline since, the arrival of domestic sheep to areas populated by this species. The decline in numbers of bighorn sheep can be partially attributed to their enhanced susceptibility to specific pathogens acquired from domestic sheep. Epizootics can have devastating consequences for bighorn sheep restoration and management of these populations. *Mannheimia* and *Pasteurella* seem to be common organisms associated with pneumonia epizootics. The reasons for the differences in susceptibility to these respiratory pathogens between domestic and bighorn sheep are not known. However, in terms of evolution of the host immune response to bacterial challenge, it is of interest to examine these two species in more detail.

There is relatively little information available comparing innate immune parameters of domestic and bighorn sheep. An examination of such parameters in domestic and bighorn sheep may provide clues as to why these species differ in susceptibility to specific bacterial infections. Previously, it was shown that bighorn and domestic sheep macrophages did not differ in phagocytic ability or in killing of *Staphylococcus epidermidis* [14]. In addition, numbers of phagocytic cells in alveolar spaces were similar between the two species and protein and cortisol levels in bronchoalveolar lavage fluid did not differ. Thus, our data showing bighorn sheep macrophages secreted significantly higher levels of nitrite than domestic sheep macrophages in response to bacterial LPS is the first reported difference between domestic and bighorn sheep related to innate immune mechanisms.

In response to bacterial recognition, cells of the mammalian innate immune system initiate a cascade of events to neutralize and/or eliminate the microorganism. There are numerous receptors recognizing pathogen-associated molecular patterns involved in activation of this innate response. For example, it has been known for some time that CD14, a 55 kDa glycosylphosphatidylinositol (GPI)-anchored protein, is a receptor for bacterial LPS. It was theorized that an additional molecule or molecules must associate with GPI-anchored CD14 in order to transduce a signal in response to LPS binding [15]. More recently, toll-like receptor (TLR)-4 and a soluble protein, MD-2, were shown to form a tri-molecular receptor with CD14 leading to LPS-induced signaling [16]. Our data indicates that bighorn and domestic sheep macrophages differ in response to *M. haemolytica* LPS stimulation in spite of the fact that they express similar levels of CD14. Whether macrophages from these two species differ with

respect to signaling via TLR-4/MD-2 and/or other LPS-binding molecules awaits further experimental investigation.

Macrophages play a critical role in the innate immune response by releasing cellular components, such as reactive nitrogen and oxygen intermediates, which act as effector molecules with cytostatic or cytotoxic activity. Expression of mammalian NOS is regulated by translational and post-translational mechanisms. NO is produced when NOS is present as a homodimer. Formation of a NOS homodimer requires multiple binding reactions and may involve at least five molecules in addition to the co-substrates and the monomeric partner [3]. Determining the mechanism(s) whereby macrophages from bighorn and domestic sheep differ in the release of NO in response to LPS stimulation will require elucidation of the regulatory pathways of NO production as have been delineated for other mammalian species.

Signaling pathways resulting in NOS expression require activation of transcription factors such as NFκB and STAT1-α [17]. For transcriptional regulation of murine iNOS following bacterial LPS stimulation, nuclear factor IL-6 (NFIL-6) and NFκB binding sites in basal enhancer region I and a NFκB binding site in region II have been identified [18]. Regulation of human iNOS seems to require NFκB as well. However, regulation of the human NOS2 (iNOS) promoter is more complex than its murine counterpart and multiple transcription initiation sites have been described [19]. NOS2 has been described in other mammalian species, including sheep, but it remains to be determined whether mechanisms of transcriptional regulation of NOS2 differ between domestic and bighorn sheep.

In some mammals, for example, humans and rats, it has been shown that the normal airway epithelium is uniformly labeled with an anti-iNOS antibody [20]. Our findings of higher levels of NO production by bighorn sheep macrophages compared to domestic sheep macrophages suggest that it would be of interest to determine if domestic and bighorn sheep airway epithelia differ in expression of NOS following respiratory infection. High levels of NO secretion by airway epithelia could further contribute to the severity of respiratory diseases in bighorn sheep.

As mentioned, an aberrant induction of NO seems to be involved in the pathophysiology of many diseases [4,5]. Furthermore, iNOS deficient mice were protected from LPS-induced cardiovascular collapse and death suggesting a role for NO in endotoxic shock [21]. It is plausible to suggest that an induction of high levels of NO could contribute to the enhanced susceptibility of bighorn sheep to *Mannheimia* infection. Thus, it will be of interest to determine in vivo whether NO plays a role in the pathophysiology of *M. haemolytica* infection in bighorn sheep.

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